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Stress-induced elevations of γ-aminobutyric acid type A receptor-active steroids in the rat brain

(allopregnanolone/allotetrahydrodeoxycorticosterone/endogenous ligands/$GABA_A$ receptors/stress)

ROBERT H. PURDY*†, A. LESLIE MORROW‡§, PERRY H. MOORE, JR.*, AND STEVEN M. PAUL‡

*Department of Organic Chemistry, Southwest Foundation for Biomedical Research, San Antonio, TX 78228; and ‡Section on Molecular Pharmacology, Clinical Neuroscience Branch, National Institute of Mental Health, Bethesda, MD 20892

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ABSTRACT **A 3α-hydroxy A-ring-reduced metabolite of progesterone, 3α-hydroxy-5α-pregnan-20-one (allopregnanolone), and one of deoxycorticosterone (DOC), 3α,21-dihydroxy-5α-pregnan-20-one (allotetrahydroDOC), are among the most potent known ligands of γ-aminobutyric acid (GABA) receptors designated $GABA_A$ in the central nervous system. With specific radioimmunoassays, rapid (<5 min) and robust (4- to 20-fold) increases of allopregnanolone and allotetrahydroDOC were detected in the brain (cerebral cortex and hypothalamus) and in plasma of rats after exposure to ambient temperature swim stress. Neither steroid was detectable in the plasma of adrenalectomized rats either before or after swim stress. However, allopregnanolone, but not allotetrahydroDOC, was still present in the cerebral cortex (>3 ng/g) after adrenalectomy. These data demonstrate the presence of allopregnanolone and allotetrahydroDOC in brain and show that acute stress results in a rapid increase of these neuroactive steroids to levels known to modulate $GABA_A$ receptor function.**

It has been almost 50 years since Selye (1) reported the anesthetic and sedative properties of several 3α-hydroxy A-ring-reduced pregnane steroids, including the major metabolites of progesterone, 3α-hydroxy-5α-pregnan-20-one (allopregnanolone) and 3α-hydroxy-5β-pregnan-20-one (pregnanolone), and the major metabolite of deoxycorticosterone (DOC), 3α,21-dihydroxy-5α-pregnan-20-one (allotetrahydroDOC). In addition to their sedative–hypnotic and anesthetic effects, these steroids have subsequently been shown to produce a number of other behavioral effects when administered to laboratory animals, including anticonflict (2), anticonvulsant (3), and analgesic actions (4). The behavioral effects of these 3α-hydroxysteroids do not appear to involve genomic events that are mediated by steroid receptors since they occur quite rapidly (seconds to minutes) after parenteral administration, and since neither steroid binds with appreciable affinity to progesterone receptors (5). Recently, we and others (6–13) have reported that allopregnanolone and allotetrahydroDOC bind with high affinity to recognition sites associated with the γ-aminobutyric acid (GABA) receptor complex. The $GABA_A$ receptor is one of two known receptors for GABA, the principal inhibitory neurotransmitter in brain, and has been shown to mediate the anxiolytic, sedative, and hypnotic actions of many drugs, including benzodiazepines and anesthetic barbiturates (14, 15). Radioligand binding and $^{36}Cl^-$ flux studies (6–12) as well as electrophysiologic experiments in cultured embryonic neurons (16) have provided evidence for the presence of unique and perhaps multiple steroid recognition sites (13) associated with the $GABA_A$ receptor macromolecular complex. In experiments using recombinantly expressed $GABA_A$ receptors, both allopregnanolone and allotetrahydroDOC were effective in potentiating GABA-activated Cl^- currents at low nanomolar concentrations, comparable to the most potent benzodiazepines (17).

The relatively high affinity of these 3α-hydroxysteroids for $GABA_A$ receptors (6–12), the presence of unique steroid recognition sites on the $GABA_A$ receptor complex (13), and the presence in brain of both 5α-steroid reductase and 3α-hydroxysteroid oxidoreductase (18–23) have prompted speculation that these 3α-hydroxysteroids are endogenous modulators of central $GABA_A$ receptors (6–12). We now report the presence of both of these natural 3α-hydroxy A-ring-reduced steroids in brain, and demonstrate that their levels are rapidly and markedly increased in the cerebral cortex, hypothalamus, and plasma of male rats for 1 hr after brief ambient temperature swim stress.

METHODS

Animals. Adult (200–250 g) male Sprague–Dawley rats (Taconic Farms) were maintained in a 12-hr light/dark cycle (light on at 0700) with free access to water and National Institutes of Health open formula no. 1 rat chow. Adrenalectomized (ADX) animals were prepared 10–14 days prior to the stress experiment. Plasma levels of corticosterone were undetectable by RIA at the time of experiment. Rats were subjected to acute stress (24, 25) by swimming for 5 or 10 min in ambient temperature water (22°C) in a cylindrical container (24″ × 36″) filled to 75% capacity. After 5 or 10 min, the rats were removed from the container, gently dried with towels, and placed in plastic cages containing bedding until the time of sacrifice. Rats were rapidly euthanized by decapitation according to guidelines of the American Association for the Accreditation of Laboratory Animal Care, and all protocols were approved by the Animal Care and Use Committee, National Institute of Mental Health. The brains were rapidly removed, dissected, and samples of cerebral cortex and hypothalamus were frozen on dry ice within 2 min after decapitation and stored at −80°C until analysis. Plasma samples were treated similarly after separation from formed elements for 90 min at 4°C.

Extraction Procedure. The plasma samples (0.3 ml) were extracted with ether as described (26). For cerebral cortex (300 mg per rat) and hypothalamus (four rats per sample, 40 mg each), tissue was homogenized at 4°C in 50% aqueous methanol (10 ml) containing 1% acetic acid with 3000 dpm of

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Abbreviations: GABA, γ-aminobutyric acid; ADX, adrenalectomized; pregnanolone, 3α-hydroxy-5β-pregnan-20-one; allopregnanolone, 3α-hydroxy-5α-pregnan-20-one; DOC, deoxycorticosterone; allotetrahydroDOC, 3α,21-dihydroxy-5α-pregnan-20-one; ANOVA, analysis of variance; HSD, Tukey's honestly significant difference procedure.

†To whom reprint requests should be addressed.
§Present address: Department of Psychiatry and Center for Alcohol Studies, University of North Carolina School of Medicine, Chapel Hill, NC 27599.

the internal recovery standard for each steroid. The homogenate was centrifuged at 1200 × *g* for 10 min at 4°C, and the supernatant was passed through a C_{18} Sep-Pak cartridge (Millipore) that had been equilibrated with homogenizing buffer. The cartridge was washed sequentially with homogenizing buffer (10 ml), water (10 ml), 50% aqueous methanol (10 ml), and the steroid fraction was eluted with absolute methanol (10 ml) and taken to dryness under a stream of nitrogen.

Chromatography. Extracts were purified by HPLC using a Waters 8-cm Radial-Pak 5-μM silica column (26) with 0.3% ethanol (95%) in dichloromethane as the initial solvent at a flow rate of 3 ml/min. Fractions were collected at 0.2-min intervals. Allopregnanolone was eluted at 9–11 min; pregnanolone, at 15–17 min; and progesterone, at 18–20 min. In experiments where allotetrahydroDOC was assayed, the initial solvent was changed at 12 min to 1.8% ethanol (95%) in dichloromethane, which resulted in the elution of allotetrahydroDOC at 18.5–20.5 min. The eluates were combined for each steroid and evaporated *in vacuo*, and an aliquot was used to determine the recovery of the tritiated internal standard (average about 70%).

RIAs. Allopregnanolone in the purified extracts was measured by RIA as described (26). The sensitivity of this assay is 25 pg, with a precision between duplicate determinations averaging 6.5% and an interassay coefficient of variation of 8.5%. A similar RIA for allotetrahydroDOC used polyclonal antibodies that were raised in a rabbit against 3α,21-dihydroxy-20-oxo-5α-pregnan-11α-yl carboxymethyl ether coupled to bovine serum albumin (unpublished results). Other cross-reacting steroids were separated from this metabolite by HPLC as described above. Neither pregnenolone nor pregnenolone sulfate cross-reacted in either assay at concentrations previously reported for the rat brain (27). Precision, measured by the interassay coefficient of variation averaged 7.3%. The intraassay coefficient of variation averaged 9.2%. The blank averaged 18.6 ± 12.1 pg, and the sensitivity of the assay was 24 pg. An RIA similar to that for allopregnanolone (26) was used to measure pregnanolone and used polyclonal antibodies raised in a rabbit against 3α,21-dihydroxy-5β-pregnan-20-one 21-hemisuccinate coupled to bovine serum albumin. For progesterone, we used an RIA kit from ICN, with antibodies raised in sheep against 11α-hydroxyprogesterone coupled to bovine serum albumin. Corticosterone was measured by RIA with a radioiodine assay kit (ICN).

Materials. [9α,11α,12α-^{3}H]Allopregnanolone (42 Ci/mmol; 1 Ci = 37 GBq), [9α,11α,12α-^{3}H]allotetrahydroDOC (61 Ci/mmol), [11,12-^{3}H(N)]pregnanolone (41 Ci/mmol), and [1,2-^{3}H]progesterone (57 Ci/mmol) were obtained from New England Nuclear. Unlabeled steroids were synthesized as described (28). Water, methanol, 95% ethanol, and dichloromethane were of HPLC grade.

RESULTS

Allopregnanolone was detectable (Fig. 1) in both the cerebral cortex (2.4 ± 0.33 ng/g) and hypothalamus (1.7 ± 1.0 ng/g) of control (nonstressed) rats (n = 24). Acute swim stress markedly increased allopregnanolone levels in the cerebral cortex and hypothalamus of male rats within 5 min after exposure to the stressor (Fig. 1). The stress-induced increases in allopregnanolone returned to baseline levels by 2 hr. Allopregnanolone was low (n = 4) or undetectable (n = 8) in the plasma of nonstressed rats but increased to about 3 ng/ml 1 hr after acute swim stress (n = 12; Fig. 1). The epimeric progesterone metabolite pregnanolone was not detectable in the plasma or cortex of these stressed animals (data not shown).

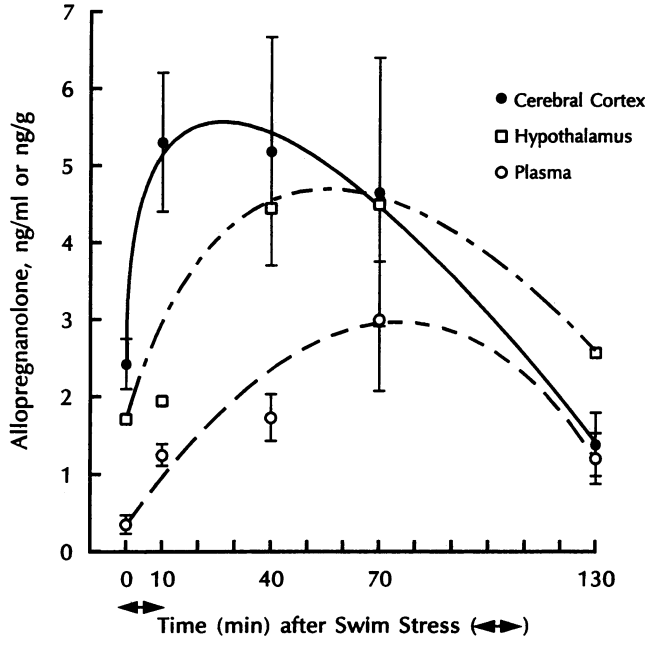

FIG. 1. Allopregnanolone levels in plasma and brain of adult Sprague–Dawley male rats after acute swim stress. Rats were subjected to acute stress by swimming for 10 min in ambient temperature water. Allopregnanolone levels were measured by RIA after purification of the steroid by HPLC. The data represent the mean ± SEM for three separate experiments (n = 12 rats per time point) for the cerebral cortex and plasma. Hypothalamic tissue was pooled for each time point and measured in duplicate. Stress-induced increases of allopregnanolone in the cerebral cortex and plasma were statistically significant at 10, 40, and 70 min after the initiation of swim stress compared with nonstressed levels at 0 time [analysis of variance (ANOVA), $P < 0.05$; Tukey's honestly significant difference procedure (HSD), $P < 0.05$].

To elucidate the origin of allopregnanolone found in the cerebral cortex and plasma before and after stress, we measured progesterone and allopregnanolone in sham-operated and ADX male rats. ADX animals were studied 10 days after adrenalectomy when plasma corticosterone levels were undetectable. As shown in Fig. 2*A*, the levels of both progesterone and allopregnanolone were increased in the cerebral cortex of sham-operated rats after swim stress ($P <$ 0.05). Adrenalectomy largely prevented the stress-induced increase in the levels of progesterone and allopregnanolone in the cerebral cortex. However, although the levels of progesterone were low to undetectable in the cerebral cortex of control and ADX rats, allopregnanolone was measurable in the cerebral cortex of both control (Fig. 2*A*) and stressed ADX rats (2.83 ± 0.69 ng/g; $P < 0.05$). In contrast to plasma, the levels of allopregnanolone in brain were equal to or greater than those of progesterone (Fig. 2).

Allopregnanolone and progesterone levels were low or undetectable, respectively, in the plasma of sham-operated control (nonstressed) male rats (Fig. 2*B*). Stress increased progesterone levels approximately 17-fold in plasma to 8.6 ± 0.3 ng/ml, while allopregnanolone levels were increased approximately 8-fold to 1.3 ± 0.3 ng/ml after swim stress. Progesterone, allopregnanolone, and corticosterone were undetectable in the plasma of both stressed ADX rats (Fig. 2*B*), and nonstressed ADX rats (data from a separate experiment, not shown). Quantitative differences in brain allopregnanolone levels have been observed between experiments performed several months apart (compare for example the data in Figs. 1 and 2*A*). However, in all experiments, swim stress consistently resulted in rapid increases in allopreg-

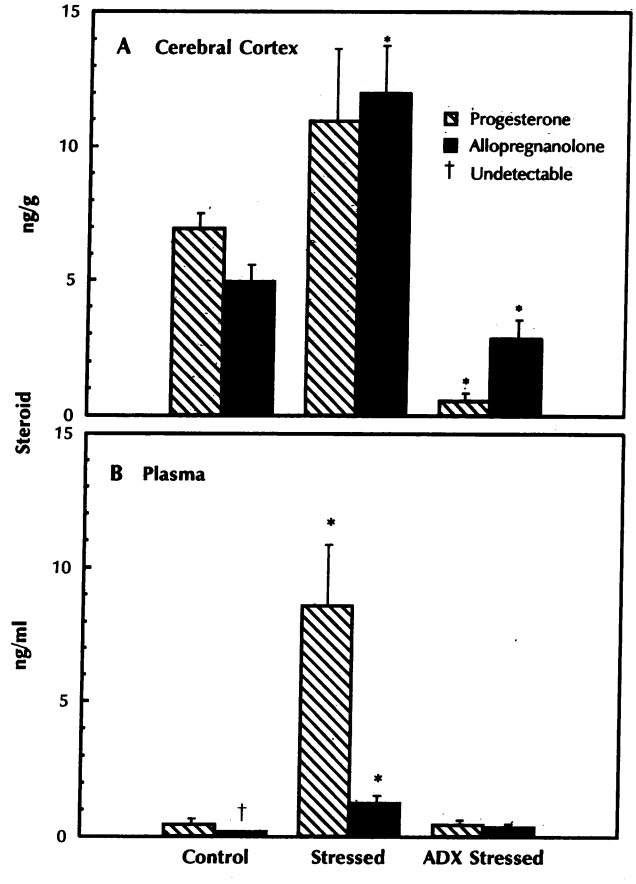

FIG. 2. Effect of swim stress on steroid levels in cerebral cortex (*A*) and plasma (*B*) of adult male rats. Animals in the control and stressed group ($n = 8$) were sham-operated 2 weeks prior to the experiment. The tissues were obtained immediately after the 10-min stressor. Allopregnanolone and progesterone levels were measured by RIA after purification of the steroids by HPLC. (*A*) In cerebral cortex the mean levels of allopregnanolone in the stressed group were significantly greater (*) than the controls (ANOVA, $P < 0.05$; HSD, $P < 0.05$). Both steroid levels in the cerebral cortex of the stressed group were significantly greater than the levels in the stressed ADX group (HSD, $P < 0.005$). (*B*) In plasma the mean levels of steroids in the stressed group were also significantly greater than in the control group (ANOVA, $P < 0.01$; HSD, $P < 0.05$) and in the stressed ADX group (HSD, $P < 0.05$ for allopregnanolone and $P < 0.01$ for progesterone).

nanolone levels in the cerebral cortex, hypothalamus, and plasma.

The $GABA_A$ receptor-active metabolite of deoxycorticosterone, allotetrahydroDOC, was measured in male rats where swim stress also markedly and rapidly increased its level in cerebral cortex and plasma (Fig. 3). Further, the level of allotetrahydroDOC in plasma was approximately twice that measured in the cerebral cortex. Adrenalectomy reduced the levels of allotetrahydroDOC in plasma and cortex to below the detection limit of our assay and completely prevented the increases observed after stress (Fig. 4).

DISCUSSION

Several laboratories have now reported that allopregnanolone and allotetrahydroDOC are high-affinity ligands of $GABA_A$ receptors (5–13) and markedly augment $GABA_A$ receptor-mediated Cl^- conductance or flux at submicromolar concentrations in a variety of broken cell (e.g., microsac, synaptoneurosome) and whole cell (e.g., cultured fetal neurons or brain slice) preparations. The affinities of allopregnanolone and allotetrahydroDOC for $GABA_A$ receptors are

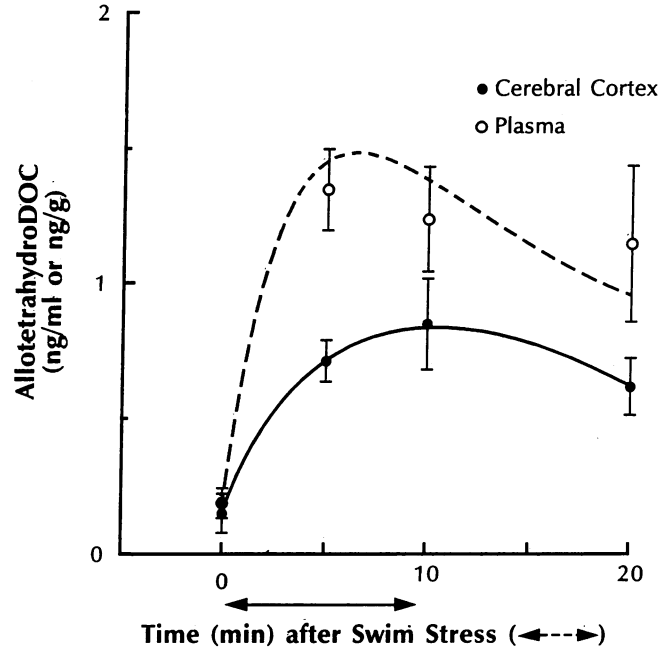

FIG. 3. AllotetrahydroDOC levels in adult male rats after acute swim stress. A group of eight normal animals were stressed for 5–10 min. AllotetrahydroDOC levels were measured by RIA after purification of the steroid by HPLC. Swim stress significantly increased allotetrahydroDOC levels in the cerebral cortex and plasma at 5, 10, and 20 min after the initiation of swim stress compared with nonstressed levels at 0 time (ANOVA, $P < 0.005$; HSD, $P < 0.05$).

comparable to that of the benzodiazepines and are thus among the most potent of the known $GABA_A$ receptor ligands (15). This, coupled with the fact that these steroids are natural metabolites of progesterone and deoxycorticosterone, has prompted considerable speculation that they are endogenous ligands (positive allosteric modulators) of $GABA_A$ receptors (5–13). To our knowledge, neither the presence of these steroids in brain nor their levels under basal or stress-induced conditions has ever been reported. The presence of allopregnanolone has been measured in ovarian blood (29, 30) and adrenal venous blood (31) by using gas–liquid chromatography; however, this method was not sensitive enough to measure this steroid in brain tissue or

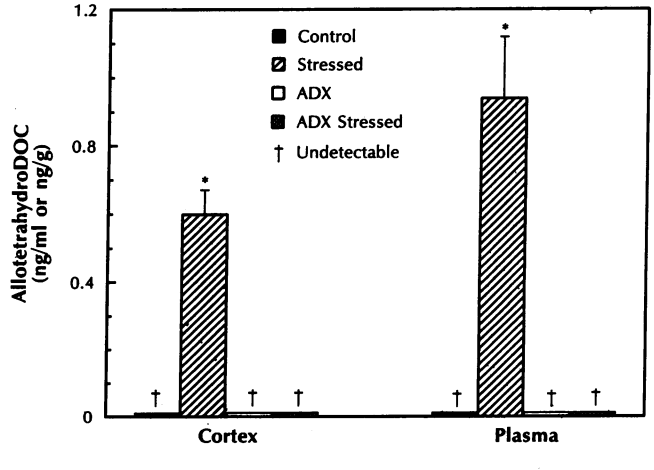

FIG. 4. Effect of swim stress and adrenalectomy on the levels of allotetrahydroDOC in the cerebral cortex and plasma of groups ($n = 8$) of the adult male rats used in the experiment shown in Fig. 2. AllotetrahydroDOC levels were measured by RIA after purification of the steroid by HPLC. The means in the stressed group were significantly greater (*) than in the control or ADX groups (ANOVA, $P < 0.005$; HSD, $P < 0.005$).

peripheral venous blood. Using recently developed RIAs, we have now identified both allopregnanolone and allotetrahydroDOC in the brains of male rats (this report) and female rats (unpublished data). In the nonstressed male rat, the brain and plasma levels of allopregnanolone and allotetrahydroDOC are relatively low (<1 ng/g or ng/ml, respectively) but rise quite rapidly in response to stress to levels (≥3–6 ng/ml, 10–20 nM) that have been shown to augment GABA responses in synaptoneurosomes (8, 13), cultured fetal hippocampal neurons (7), chromaffin cells (32), and human embryonic kidney cells with recombinantly expressed $GABA_A$ receptors (17). Thus, the levels of allopregnanolone and allotetrahydroDOC in the male rat brain appear to be sufficient to affect $GABA_A$ receptor-mediated inhibitory events.

Experiments designed to delineate the source of brain and plasma allopregnanolone and allotetrahydroDOC have revealed that the major, if not exclusive, source of allotetrahydroDOC is the adrenal gland (Fig. 3). We have previously reported that the plasma/brain ratio of allotetrahydroDOC is 1.47 ± 0.05 when measured 20 min after i.v. administration of ^{3}H-labeled allotetrahydroDOC to male rats (28). Given that allotetrahydroDOC is significantly more polar than allopregnanolone, the higher level of this steroid in plasma compared with brain is not unexpected. The failure to detect allotetrahydroDOC in the plasma and brain of control, ADX, or ADX stressed rats (Fig. 3) strongly suggests that this metabolite is formed entirely by the adrenal gland in this species, since we were unable to detect the conversion of allopregnanolone to allotetrahydroDOC in the rat brain (28). By contrast, allopregnanolone was still measurable (≥3 ng/g or 100 times the minimal detectable level of 25 pg/g) in the cerebral cortex of ADX rats (Fig. 2). Allopregnanolone has recently been shown to be formed *in vitro* from progesterone by cultured fetal glial cells (23). The hypothesis that allopregnanolone biosynthesis occurs in brain from progesterone formed *in situ* is supported by the following observations. First, allopregnanolone is detectable in the cerebral cortex and hypothalamus of control (nonstressed) rats even in the absence of detectable levels of circulating allopregnanolone (Fig. 1). Second, the peak levels of allopregnanolone in cerebral cortex observed following swim stress occur 30–60 min prior to the peak levels in plasma (Fig. 1). Finally, allopregnanolone is still measurable in the cerebral cortex of ADX rats even when plasma progesterone levels are undetectable (Fig. 2). The latter would also argue strongly against the possible contribution of other peripheral sources (i.e., the testes) of progesterone or allopregnanolone to the brain levels measured in ADX rats.

In intact male rats, the levels of allopregnanolone in the cerebral cortex are equal to or greater than that of the parent steroid progesterone (Fig. 2). However, in ADX rats (and especially after swim stress), the level of allopregnanolone in brain greatly exceeds that of progesterone (Fig. 2). The latter observation suggests that a substantial fraction of the progesterone formed in brain is rapidly converted to allopregnanolone. Recently, Hall and colleagues have identified a protein that is essential for the ACTH or cyclic AMP-induced stimulation of steroidogenesis in the adrenal cortex (33). Interestingly, this protein is virtually identical in amino acid sequence to diazepam binding inhibitor (DBI), a protein previously isolated by Guidotti and colleagues and shown to be an endogenous ligand of both central and peripheral benzodiazepine recognition sites (34). Since the peripheral benzodiazepine receptor also has been shown to regulate adrenal steroidogenesis (35, 36), it is tempting to speculate that DBI may regulate the synthesis of allopregnanolone in the adrenal gland and/or brain (37). Given the demonstrated ability of glial cells to convert cholesterol to pregnenolone (20, 38, 39), to transform pregnenolone to progesterone (20), and to synthesize allopregnanolone from progesterone (23), it is possible that progesterone is formed in glia from pregnanolone and then rapidly reduced by glia and/or neurons to allopregnanolone (22), thus accounting for the basal levels of allopregnanolone found in the cerebral cortex of adrenalectomized rats.

Alternatively, release of allopregnanolone from steroid binding sites in brain (40) or hydrolysis of fatty acid esters of allopregnanolone stored in brain (39) might account for the increased levels of this metabolite found in brain after stress. We consider these alternative explanations to be unlikely and believe that the stress-induced increases of allopregnanolone in brain are predominately of adrenal origin.

The stress-induced increases in the brain and plasma levels of allopregnanolone and allotetrahydroDOC may have important behavioral and/or neuroendocrine consequences. These steroids have been shown to produce anticonflict, analgesic, and anticonvulsant actions in rodents (2–4). Such behavioral effects may represent an adaptive response of the organism to stress. Moreover, GABA has been shown to inhibit prolactin release from the pituitary, and this inhibitory effect is selectively potentiated by allopregnanolone but not by progesterone (41). GABA has also been shown to inhibit the release of corticotropin-releasing factor (CRF) from the hypothalamus *in vitro* (42). Thus, stress-induced increases in allopregnanolone could result in a diminished release of CRF and thus ACTH/corticosterone. The latter may represent a novel feedback loop for decreasing the heightened activity of the hypothalamic–pituitary–adrenal axis after stress.

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